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The Mechanism of the Aminoacylation of Transfer Ribonucleic Acid: Enzyme·Product Dissociation Is Not Rate Limiting[†]

Timo Nils E. Lövgren,[‡,§] Andrzej Pastuszyn, and Robert B. Loftfield*

ABSTRACT: It has been proposed that the rate-limiting step in the synthesis of aminoacyl-tRNA is the rate at which the product dissociates from the enzyme. The experimental evidence supporting this hypothesis comes from work at low pH and low temperature (although the reaction has been argued to have the same mechanism under physiological conditions). We have reexamined the binding assay by which M. Yarus and P. Berg (1969) (*J. Mol. Biol. 42,* 171-189) measured the k_d for dissociation of Enz·(Ile-tRNA). We find that when overall reaction and dissociation are measured under identical conditions the two rates are not the same. Moreover, while an increase in ionic strength greatly stimulates dissociation, the same increased ionic strength slows aminoacylation. Spermine accelerates overall aminoacylation without affecting dissociation. Because any change in a rate-limiting step must, by definition, cause a parallel change in the overall reaction, these observations prove that under these conditions the synthesis of Ile-tRNA is not limited by the rate of dissociation of Enz·(Ile-tRNA). Entirely similar observations were made for the dissociation of Enz·(Val-tRNA) and the overall synthesis of Val-tRNA at 0 °C, pH 5.0. In addition, valine enzyme isolated by nitrocellulose filtration during the course of an aminoacylation was shown not to be saturated with recently synthesized Val-tRNA. The enzyme was in equilibrium with uncharged substrate tRNA and with product Val-tRNA. E. W. Eldred and P. R. Schimmel ((1972) *Biochemistry 11,* 17-23) report that the formation of Ile-tRNA proceeds at two rates: (a) $k = 2 \times 10^{-2}\ \text{s}^{-1}$ until the enzyme is saturated with the first mole of product, and (b) $k = 2 \times 10^{-3}\ \text{s}^{-1}$ for subsequent cycles. We did not observe this behavior at any pH or temperature with four different amino acid:tRNA ligases. Because aminoacylation proceeds more rapidly than "dissociation" under some conditions, we believe that the binding assay measures not only enzyme·product dissociation but also other slower reactions such as aggregation or disaggregation of Enz·(AA-tRNA). In conjunction with recent studies from other laboratories, this work makes it unlikely that enzyme·product dissociation is the rate-limiting step in the synthesis of aminoacyl-tRNA either at low temperature and pH or under more nearly physiological conditions. From the effect of salt, it would appear that the rate of aminoacylation of tRNA is largely limited by the rate or extent of formation of Enz·(tRNA) (Loftfield, R. B., and Eigner, E. A. (1967), *J. Biol. Chem. 242,* 5355-5359). Using the binding assay of M. Yarus ((1972) *Biochemistry 11,* 2050-2060), we find the K_{ass} for Enz·(Ile-tRNA) varies linearly with the Debye-Hückel function at ionic strengths of 0.1-0.4 from 10^8 to 10^6.

It has been proposed that the rate-limiting step in the synthesis of several aminoacyl-tRNAs (AA-tRNA[1]) is the separation of the product from the enzyme (Yarus and Berg, 1969; Hélène et al., 1971; Eldred and Schimmel, 1972, 1973). If a particular step is rate limiting, a change in rate of that step should change the overall reaction in a parallel way.

It seemed probable that raising the ionic strength would accelerate dissociation (Pingoud et al., 1973; Krauss et al., 1973; Loftfield and Eigner, 1967); therefore, we sought to determine, under Yarus and Berg's binding assay conditions, the effect of ionic strength on the overall reaction. In general under other conditions, it is known that higher ionic strengths slow aminoacylation reactions (Loftfield and Eigner, 1967; Taglang, et al., 1970; Smith, 1969; Holten and Jacobson, 1969; Yarus, 1972; Loftfield, 1972). The only reported exception to this generalization involves the tRNA and enzymes derived from halophilic bacteria (Griffiths and Bayley, 1969). Similarly, it was likely that spermine would accelerate the overall reaction (Igarashi et al., 1971; Pastuszyn and Loftfield, 1972), so we examined the effect of spermine on dissociation.

[†] From the Department of Biochemistry, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131. *Received December 31, 1975.* This work has been supported by United States Public Health Grant CA-08000 and by American Cancer Society Grant NP103D. A preliminary report of this work has been made (Pastuszyn et al., 1974).

[‡] Present address: Institutionen for Biokemi och Farmaci, Åbo Akademi, 20500 Åbo, Finland.

[§] Recipient of a travel stipend from Finlands Akademi Naturvetenskapliga Kommissionen.

[1] Abbreviations used are: tRNA$^{\text{AA}}$, unesterified transfer ribonucleic acid specific for a particular amino acid; AA-tRNA, transfer ribonucleic acid esterified with its specific amino acid; Enz, free enzyme (amino acid:tRNA ligase specific for a particular amino acid (EC 6.1.1)); AA$\sim$AMP aminoacyl adenylate; Enz·(tRNA$^{\text{Ile}}$), Enz·(Val-tRNA), Enz·(AA$\sim$AMP), etc., ligase bound to tRNA$^{\text{Ile}}$, to Val-tRNA, to aminoacyl adenylate etc., respectively; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; GSH, reduced glutathione.

Experimental Section

Materials

Frozen *Escherichia coli* B paste was purchased from the Grain Processing Co, Muscatine, Iowa. It was stored at −20 °C until use. ATP, glass beads, streptomycin sulfate, and mercaptoethanol were purchased from Sigma; L-isoleucine, L-valine, and L-leucine were from Nutritional Biochemical Corp; [U-^{14}C]isoleucine, [U-^{14}C]arginine, [U-^{14}C]valine, and [U-^{14}C]leucine were from Amersham; dithioerythritol was from Pierce Chemical; Sephadex was from Pharmacia; DEAE-cellulose and cellulose were from Whatman; hydroxylapatite was from Bio-Rad; and membrane filters were from Schleicher and Schuell or from the Millipore Corp.

tRNA$^{\rm Ile}$, tRNA$^{\rm Leu}$, tRNA$^{\rm Arg}$, and tRNA$^{\rm Val}$ were prepared from unfractionated *E. coli* B tRNA (Schwarz Bioresearch) according to the method of Gillam et al. (1967). The purified tRNA's had acceptor activities in the range of 1.7–1.8 nmol/A_{260} unit at pH 7.0. Each tRNA was treated with Chelex resin and by dialysis against EDTA, after which the residual Mg^{2+} was less than 2 atoms of Mg^{2+}/molecule of tRNA.

Isoleucine:tRNA ligase (isoleucyl-tRNA synthetase) was prepared from *E. coli* paste by published methods (Lövgren, et al., 1975). The enzymes specific for valine, arginine, and leucine separated during DEAE-cellulose chromatography and were subsequently purified by the methods used for the isoleucine enzyme. Enzyme concentrations were determined by measurement of Enz·(AA∼AMP) isolated after Sephadex filtration (Yarus and Berg, 1970) and by titration against [^{14}C]AA-tRNA in the binding assay. The two assays agreed within 30%.

[^{14}C]Ile-tRNA$^{\rm Ile}$ was prepared by incubating together at 27 °C the following in 300 μl: 4 nmol of tRNA$^{\rm Ile}$, 100 μM [^{14}C]isoleucine (240 cpm/pmol), 3.3 mM ATPMg, 4 mM GSH, 0.03% albumin, 20 mM Tris (pH 7.6), 50 mM KCl, and 12 pmol of isoleucine enzyme. After 20 min, conversion to [^{14}C]Ile-tRNA was complete, and the product was precipitated by addition of two volumes of ethanol, collected, washed twice with 0.4 ml of cooled 80% ethanol, and redissolved in water. The yield, as measured by acid precipitation and radioassay, was 88%. [^{14}C]Val-tRNA$^{\rm Val}$ was similarly prepared in 92% yield.

Methods

Binding Assay (Yarus and Berg, 1970). The reaction mixture at 0 °C contained in 1.0 ml total volume: 50 mM potassium phosphate buffer, pH 5.5, 10 mM $MgCl_2$, 100 μM dithioerythritol, 100 μM EDTA, 10 μg of bovine serum albumin, and typically 84 nM [^{14}C]Ile-tRNA (240 cpm/pmol) and 100 nM isoleucine enzyme. Other components such as isoleucine, ATP, KCl, spermidine, etc., were included as indicated in the text. An aliquot was removed after mixing, another aliquot was removed after adding salt (Figure 3), and exchange was initiated by adding 480 pmol of tRNA$^{\rm Ile}$ in 5 μl of water. Thereafter aliquots of 100 μl were removed at appropriate intervals, filtered through the S and S prewashed membrane filter (No. B-6) at 0 °C, and washed with 3 ml of wash fluid (50 mM potassium phosphate buffer, pH 5.5, 50 mM $MgCl_2$). The filters were dried and assayed in a Nuclear Chicago low background Geiger counter. The Val-tRNA:enzyme studies were done in the same fashion but at pH 5.0 with 0.05 M acetate buffer instead of phosphate.

As defined by Yarus and Berg (1967), the efficiency of the binding assay was between 75 and 80% for the isoleucine system. That is, with enzyme excess, about 80% of the [^{14}C]Ile-tRNA was retained on the filter over a wide range of [^{14}C]-Ile-tRNA concentrations. The efficiency of binding for the Enz·(Val-tRNA) system was 50%. The determination of association constants at 0 °C was by the method of Yarus (1972) but at several concentrations of tRNA and enzyme.

Aminoacylation Assays (Eigner and Loftfield, 1974). The reaction was conducted in 500 μl in the same solutions as the binding assay except that no [^{14}C]AA-tRNA was present, concentrations of tRNA and enzyme were slightly different, and [^{14}C]AA was used. An aliquot was removed and reaction was initiated (unless otherwise indicated) by addition of ATP last. Aliquots of 45 μl were removed at appropriate times and expressed into 5% trichloroacetic acid. After filtration on Millipore membranes, washing with trichloroacetic acid, and drying, the samples were assayed in a low background Geiger counter.

Data Treatment. In the binding assays the radioactivity observed ranged from 200 to 3000 cpm/aliquot. Background counts (3–4 cpm) and enzyme-free blanks (10–15 cpm) were subtracted. In the aminoacylation assays, observed radioactivity ranged from 10 cpm to 1500 cpm/aliquot. A background count of 3 cpm was subtracted, but the zero time (no ATP) blank was not. As indicated in the figures, the drawn curves are least-squares approximations (exponential or linear) to the raw data. It is important to note that assays conducted at 0 °C were carried out in a cold room at 3 °C or, if conducted in a 22 °C room, the pipettes used for aliquoting were kept chilled at 0 °C. Substantial artifacts result from the use of warm pipettes.

Results

The binding assay gave results substantially identical with those reported by Yarus and Berg for similar conditions at 17 °C and pH 5.5. With the isoleucine system, the efficiency of binding was 75–80%. In the presence of 100 μM isoleucine and 100 μM ATP (Figure 1A), the observed k_d for dissociation of Enz·([^{14}C]Ile-tRNA) is 0.0093 s^{-1}, intermediate between the reported values of 0.0075 s^{-1} (no ATP, no isoleucine), and 0.043 s^{-1} (1 mM isoleucine, 1.9 mM ATP) (Yarus and Berg, 1969). However, under conditions where the concentrations of total tRNA, enzyme, isoleucine, ATP, and all other components were the same, the initial rate of aminoacylation was 0.058 mol of Ile-tRNA formed s^{-1} mol^{-1} of enzyme (Figure 1B), about six times more rapid than the putative rate-determining step. Yarus and Berg (1969) report the k_3 for overall product formation at 17 °C to be 0.05 s^{-1} under similar conditions except that they may have used 10^{-5} M isoleucine, 2 mM ATP, and 2 μM tRNA$^{\rm Ile}$. Under our conditions, the sixfold discrepancy between overall rate and "dissociation" would be interpretable as eliminating "dissociation" of this type as an obligatory step in the synthesis of isoleucyl-tRNA. However, since there are many potential sources of error in determining these rate constants, we felt it was desirable to proceed with our plan to try to modify independently the rate of dissociation and the rate of overall reaction. Nevertheless, because this comparison of rates at 17 °C is inconsistent with a widely held view of the aminoacylation reaction, we detail the experimental conditions in Figure 1.

As Yarus and Berg (1969) note, the rates of "dissociation" at 17 °C are sufficiently fast as to be difficult to determine. As we anticipated, these rates increase many fold with moderate increases in ionic strength, making accurate determinations impossible. Therefore, further comparisons of the rates of synthesis and dissociation of Enz·(Ile-tRNA) were conducted

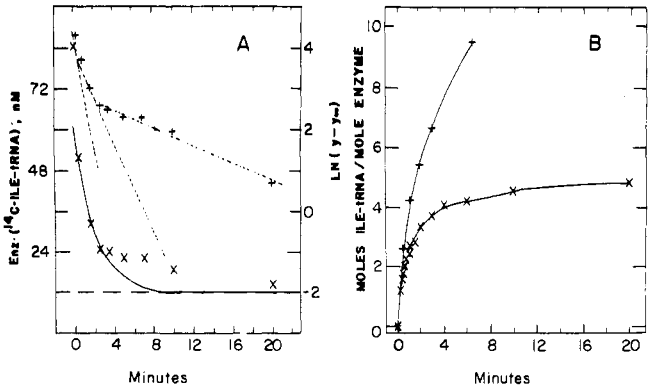

FIGURE 1: Comparison of the rates of dissociation of Enz·(Ile-tRNA), as measured by the binding assay, and synthesis of Ile-tRNA under binding conditions at 17 °C. Panel A: Dissociation. 84 pmol of [^{14}C]Ile-tRNA was combined with 100 pmol of isoleucine:tRNA ligase in 1.0 ml of solution containing 0.05 M K_2HPO_4, 0.01 M $MgCl_2$, 10 μM dithioerythritol, 0.1 μM EDTA, 0.002% bovine serum albumin, 100 μM isoleucine, and 100 μM ATP · Mg, pH 5.5. At time zero, 480 pmol of tRNA^{Ile} was added. Aliquots of 100 μl were removed and filtered, as described by Yarus and Berg (1969). The left scale and (x—x) indicate moles of [^{14}C]Ile-tRNA bound/100 mol of enzyme; the curve is the best fitting approximation to the second, third, and fourth points of the form $y - y_\infty = y_0e^{-kt}$. The dashed line is the theoretical equilibrium. The right scale and (+ · · · +) are a plot of ln ($y - y_\infty$) vs. time. The straight lines are the best approximations to the first two points, to the second-to-fourth points, and to the last six points. These emphasize the nonideality of the data. k_d (apparent) from the middle curve is 0.0092 s^{-1}; appropriately corrected, k_d = 0.0082 s^{-1}. Panel B: (x—x) Conditions exactly the same as in panel A except that no [^{14}C]Ile-tRNA was present, tRNA^{Ile} is 0.48 μM, and the isoleucine was radioactive (35 cpm/pmol). Reaction was initiated by the addition of ATP. Aliquots of 45 μl were removed and assayed for [^{14}C]Ile-tRNA as described. (+—+) Conditions exactly as above except that the tRNA concentration is five times higher (2.4 μM). The units on the ordinate are mol of Ile-tRNA formed/mol of enzyme; k_3 is approximately 0.055 s^{-1} from each curve.

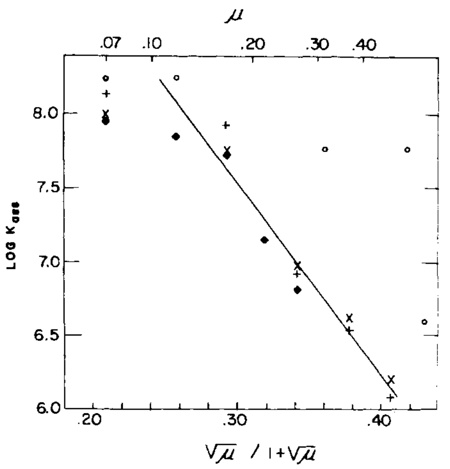

FIGURE 2: Dependence of K_{ass} for Enz · (Ile-tRNA) on ionic strength at 0 °C, pH 5.5, binding conditions. Reaction mixtures contained 75 nM enzyme and 31 nM Ile-tRNA, (x); 75 nM enzyme and 6.2 nM Ile-tRNA, (+); or 72 nM Ile-tRNA and 100 nM enzyme (◆). (This most nearly approximates the conditions for aminoacylation and dissociation studies). In addition to the salts of the standard binding assay ($\mu \simeq 0.07$), ionic strength was increased by additions of KCl. Assay and calculations as by Yarus (1972). The drawn line is the best fitting line for all data for $\mu >$ 0.07.·(Determination of K_{ass} greater than 10^8 is extremely imprecise.) For comparison, Yarus' data for association at 17 °C are also shown (○).

TABLE I: Comparison of the Rate of Dissociation of Enz·(Ile-tRNA), as Measured by the Binding Assay Method, pH 5.5, and Ile-tRNA Formation Under the Same Conditions.

	0 ATP, 0 Ile k_d (s^{-1})	10^{-4} M ATP, 10^{-4} M Ile k_d (s^{-1})	Acylation[a] k_3[b]	k_3[c]
17 °C, no added salt	0.0051	0.0093	0.058	
0 °C, no added salt	0.0011	0.0024	0.0038	0.0036
+0.05 M KCl		0.0035		
+0.10 M KCl	0.0051	0.0067	0.0018	0.0018
+0.15 M KCl		0.0138		
+0.20 M KCl	0.016	0.0195	0.00103	0.0009
+0.5 mM spermine HCl	0.0012	0.0022	0.0056	

[a] Each value is the average of three different determinations of initial rate. [b] [tRNA^{Ile}] = 0.48 μM. [c] [tRNA^{Ile}] = 2.4 μM.

at 0 °C, but otherwise under the conditions of the binding assay.

At 0 °C, the efficiency of binding was found to be 75–82%, comparable to the efficiency at 17 °C. Comparison of the amount of [^{14}C]Ile-tRNA bound 15 s after mixing the components with the amount bound after 10 min showed there were no time dependent changes. The K_{ass} for the binding of [^{14}C]Ile-tRNA with enzyme was about $10^{8.2}$ and not sensitive to variations in the concentrations or proportions of Ile-tRNA or enzyme. K_{ass} is plotted as a function of ionic strength in Figure 2; the data are comparable with the less extensive data of Yarus at 17 °C.

In order that "dissociation" and aminoacylation could be compared, dissociation was determined under aminoacylation conditions, i.e., in the presence of isoleucine and ATP, as well as enzyme, tRNA, and Ile-tRNA. Under our conditions there was no detectable hydrolysis of [^{14}C]Ile-tRNA or exchange of [^{14}C]Ile-tRNA with [^{12}C]isoleucine.

When excess tRNA^{Ile} was added to Enz·([^{14}C]Ile-tRNA), the amount of ^{14}C retained on the filter decreased exponentially with time. In the absence of isoleucine, ATP, or added salt, the k_d is 0.0011 s^{-1} (0 °C) compared with Yarus and Berg's (1969) value of 0.0075 s^{-1} (17 °C). The presence of 100 μM ATP and 100 μM isoleucine accelerated the dissociation approximately twofold at 0 °C. Increased ionic strength accelerated the dissociation ten times more (Figure 3, Table I).

When the logarithm of k_d is plotted against the Debye–Hückel function, $\mu^{1/2}/(1 + \mu^{1/2})$, a straight line with a positive slope of 9.5 is obtained. (In comparison, the data of Pingoud et al. (1973) for dissociation of Enz·Ser-tRNA at pH 7.2 also show a Debye-Hückel slope of +9.5). Spermine and spermidine, which accelerate aminoacylation reactions, do not affect "dissociation" as measured in this assay (Table I).

Many observers have noted that the overall rate of aminoacylation of tRNA is markedly lower as ionic strength is increased. However, such studies have not been conducted under the conditions of the binding assay. Using, as nearly as possible, the same conditions as we used for measuring rates of "dissociation", we determined the rate of aminoacylation as a function of ionic strength (Figure 4). In the absence of added salt at 0 °C, isoleucyl-tRNA forms at a rate of 0.0038 mol/mol of enzyme s^{-1}, rather close to the value of 0.0024 s^{-1} obtained for k_d. However, successive additions of KCl decrease the rate of aminoacylation; as noted above, increased ionic strength facilitates "dissociation". Under similar conditions, but with five times more tRNA^{Ile}, the initial rates of reaction are the same and the reaction continues at a nearly constant rate for hours. This indicates that the rate is independent of

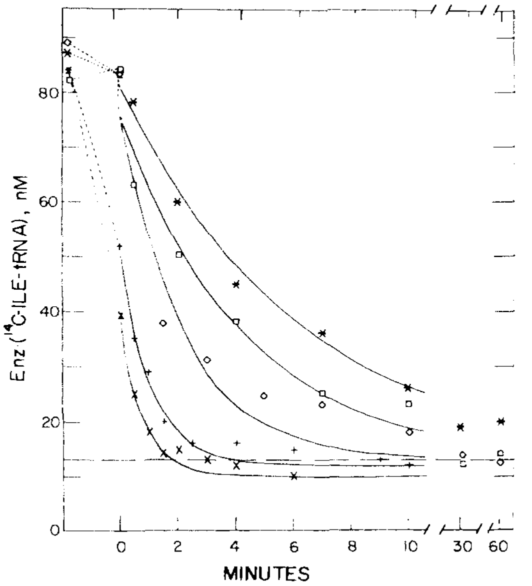

FIGURE 3: Effect of KCl on dissociation of Enz · (Ile-tRNA) under binding conditions at 0 °C, 100 nM enzyme, 84 nM of [^{14}C]Ile-tRNA, 100 μM isoleucine, 100 μM MgATP were combined in 900 μl of binding mixture (pH 5.5, 0 °C). A 90-μl aliquot was removed at −110 s. Binding solution (90 μl) containing the appropriate concentration of KCl was added and an aliquot of 100 μl was removed at −10 s. At zero time 430 pmol of tRNA$^{\text{Ile}}$ in 9 μl was added. Aliquots of 100 μl were removed at the indicated times and assayed. The ordinate indicates mol of bound [^{14}C]Ile-tRNA/mol of enzyme corrected for 80% binding efficiency. The dashed line is the theoretical equilibrium binding. The drawn curves are the least-squares best fitting curves (to the earlier time points) of the form $y - y_\infty = y_0 e^{-kt}$: (*) 0.05 M phosphate; (□) plus 0.05 M KCl; (◇) plus 0.1 M KCl; (+) plus 0.15 M KCl; (x) plus 0.2 M KCl.

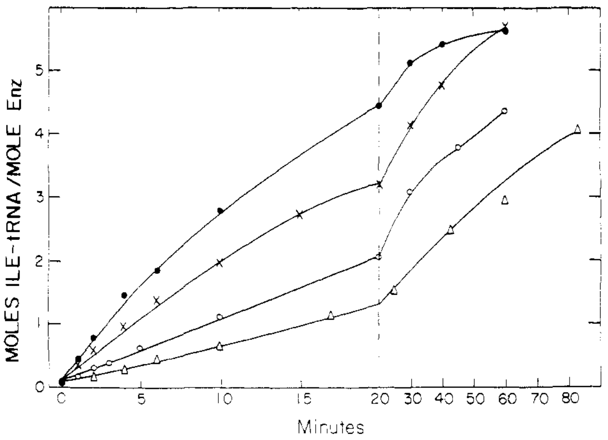

FIGURE 4: Dependence of rate of formation of Ile-tRNA on salt or spermine pH 5.5, 0 °C. Aminoacylation was conducted under binding conditions exactly like the experiments described in Figure 3 except that no Ile-tRNA was present, tRNA$^{\text{Ile}}$ was 570 nM, and the isoleucine was radioactive. Reaction was initiated by the addition of ATP. (x) Standard binding conditions; (●) plus 0.5 mM spermine HCl; (O) plus 0.1 M KCl; (△) plus 0.2 M KCl. Initial rates were determined from the straight line best fitting all the data up to the synthesis of 2 mol of Ile-tRNA/mol of enzyme. The ordinate indicates the number of mol of Ile-tRNA synthesized/mol of enzyme.

[tRNA$^{\text{Ile}}$] and that there is little or no deterioration of enzyme taking place. Furthermore, it should be noted that there is no marked change in rate corresponding to the synthesis of one molecule of Ile-tRNA/molecule of enzyme.

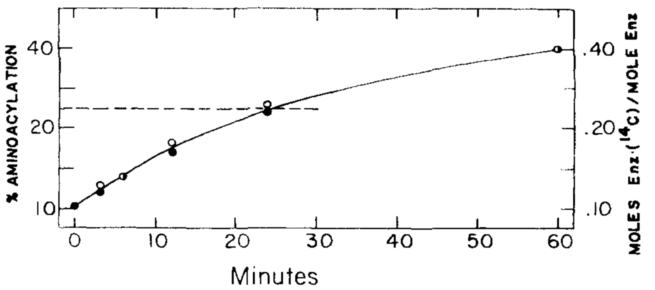

FIGURE 5: Simultaneous determination of formation of [^{14}C]Val-tRNA and amount of Enz · ([^{14}C]Val-tRNA). In 1.52 ml of a 0.05 M sodium acetate–acetic acid buffer at pH 5.0, 0 °C, were combined 0.1 mM dithioerythritol, 0.1 mM EDTA, 0.002% bovine serum albumin, 10 mM MgCl$_2$, 5 μM [^{14}C]valine, 0.3 mM ATP·Mg, and 0.2 μM valine enzyme. After 5 min at 0 °C, reaction was initiated by the addition of 1130 pmol of tRNA$^{\text{Val}}$ in 15 μl. Aliquots (40 μl) were removed and assayed for total [^{14}C]Val-tRNA by trichloroacetic acid precipitation; 200-μl aliquots were taken for the membrane filter assay. (O—O) and left scale shows the percentage of the total tRNA$^{\text{Val}}$ that has been aminoacylated. (●—●) and right scale shows the number of mol of [^{14}C]Val-tRNA bound/mol of enzyme in the binding assay: these latter data are corrected for 50% binding efficiency (i.e., each observed value has been multiplied by 2.0). The dashed line corresponds to the synthesis of 1 mol of [^{14}C]Val-tRNA/mol of enzyme

TABLE II: Comparative Rates of Dissociation of Enz·(Val-tRNA) and of Synthesis of Val-tRNA at pH 5.0, 0 °C.

	k_d (s^{-1})	k_3 (synthesis) (s^{-1})
No added salt	0.03	0.043
+0.10 M KCl	0.10	0.033
+0.20 M KCl	0.4	0.021

Spermine at 0.5 mM increases the rate of formation of isoleucyl-tRNA under binding conditions at 0 °C by about 50% (Figure 4). The initial rates of aminoacylation at varying ionic strengths are independent of tRNA$^{\text{Ile}}$ concentration (Table I).

When we attempted to measure the rate of dissociation of Enz·[^{14}C]Val-tRNA$^{\text{Val}}$ by the Yarus and Berg (1969) method, we found the rates were inconveniently high even at pH 5.5 and 0 °C. (Hélène et al. (1971) record rates from 0.004 to 0.04 s^{-1} at 20 °C.) Reducing the pH to 5.0 slowed the rates of aminoacylation and dissociation sufficiently to show that the effect of salt on the two reactions was opposed (Table II). However, because the observations are in conflict with the conclusions of Hélène, we attempted another test. If the aminoacylation reaction proceeds rapidly to the formation of Enz·([^{14}C]Val-tRNA) and then more slowly to release [^{14}C]Val-tRNA, one would expect that the amount of Enz·([^{14}C]Val-tRNA) isolated in the binding assay would increase rather rapidly with time until one molecule of [^{14}C]valine was bound/molecule of enzyme; thereafter the ^{14}C measured in the binding assay would remain constant. The total [^{14}C]Val-tRNA formed (as measured by trichloroacetic acid precipitation) would increase rapidly during the first cycle of synthesis and thereafter would increase more slowly. On the other hand, if dissociation of product from the enzyme is rapid compared to synthesis of Val-tRNA, one would expect that the amount of tRNA bound to enzyme in the binding assay would reflect the relative proportions of unacylated tRNA$^{\text{Val}}$ and [^{14}C]Val-tRNA. In other words, the specific activity of the Enz·tRNA complex would increase in rough proportion to the extent of conversion of tRNA to Val-tRNA. Figure 5 demonstrates that the enzyme is never saturated with freshly synthesized [^{14}C]Val-tRNA; the filtered enzyme is always in equilibrium with the mixture of substrate tRNA and product Val-tRNA.

Discussion

As noted in Figure 1 and Table I, the formation of isoleucyl-tRNA at 17 °C and pH 5.5 is about six times more rapid than the "dissociation" of Enz·(Ile-tRNA) under the same conditions. The conditions differ only slightly from those used by Yarus and Berg (1969) who reported aminoacylation at about our rate and dissociation six times as fast, the chief difference being that they used higher concentrations of ATP and isoleucine. If there are any conditions under which the rate of the overall reaction is greater than the apparent rate of an obligatory intermediate step, it is likely that the rate of the intermediary reaction has not been correctly determined.

The effects of variations in ionic strength at 0 °C confirm the suspicion that "dissociation", as measured by the binding assay, is not directly related to the rate-determining step of aminoacylation. Figures 3 and 4 and Table I show that higher ionic strengths, which systematically increase the rate of "dissociation", systematically slow the overall reaction; that one reagent spermine, which accelerates aminoacylation, has no effect on "dissociation". By definition, one expects that any change in rate of the rate-determining step will result in a similar change in the rate of the overall reaction until conditions are changed so much as to make another step rate-limiting.

In this case, two sets of experiments are absolutely contrary to the expectation. Kinetic analysis emphasizes the paradox.

Eldred and Schimmel (1972) proposed the following sequence of events and rate constants (pH 6.0, 3 °C for the isoleucine enzyme system).

$$\text{Enz} + \text{ATP} + \text{AA} \rightarrow \text{Enz}\cdot(\text{AA}{\sim}\text{AMP}) + \text{PP}_i \qquad k > 10^{-1}\ \text{s}^{-1} \tag{1}$$

$$\text{Enz}\cdot(\text{AA}{\sim}\text{AMP}) + \text{tRNA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Enz}\cdot(\text{AA}{\sim}\text{AMP})\cdot\text{tRNA} \tag{2}$$

$$\text{Enz}\cdot(\text{AA}{\sim}\text{AMP})\cdot\text{tRNA} \xrightarrow{k_3} \text{Enz}\cdot(\text{AA-tRNA}) + \text{AMP} \qquad k_3 = 1.8 \times 10^{-2}\ \text{s}^{-1} \tag{3}$$

$$\text{Enz}\cdot(\text{AA-tRNA}) \xrightarrow{k_d} \text{Enz} + \text{AA-tRNA} \qquad k_d = 2 \times 10^{-3}\ \text{s}^{-1} \tag{4}$$

If we assume that the above sequence is correct and that enzyme freed from product is instantly saturated with aminoacyl adenylate (Eldred and Schimmel (1972) and Yarus and Berg (1969) propose that new aminoacyl adenylate is formed *before* product release), we may derive this rate expression (see Appendix):

$$\frac{dP}{dt} = v = \frac{k_3[\text{E}][\text{tRNA}]}{(k_2 + k_3)/k_1 + [\text{tRNA}](1 + k_3/k_d)} \tag{5}$$

$$V_{\max} = \frac{k_3[\text{E}_0]}{1 + k_3/k_d} = \frac{k_d[\text{E}_0]}{1 + k_d/k_3} \tag{6}$$

If dissociation is rate limiting, k_d is by definition smaller than k_3. Inspection of eq 6 shows that there can be no circumstances under which an increase in k_d will not cause a corresponding increase in $V_{\max}$. Using the rate constants suggested by Eldred and Schimmel (1972), $V_{\max}$ would increase threefold as k_d increases fourfold. Although there are many reports that the aminoacylation of tRNA is inhibited strongly by increases in ionic strength, most such studies have not been conducted under conditions where it is possible to separate effects on K_m and $V_{\max}$. We have previously reported that $V_{\max}$ for the formation of Val-tRNA (at pH 7.5, 25 °C) is almost insensitive to ionic strength. Yarus (1972) reports that the formation of Ile-tRNA under binding conditions (pH 5.5, 17 °C) is inhibited some 10–40% by 0.25–0.5 M KCl; since, in this case, the concentration of tRNA was high relative to K_d for Enz·tRNA, this is probably a decrease in $V_{\max}$. Table I shows that under binding conditions at 0 °C the initial rate of formation of Ile-tRNA is not sensitive to a fivefold change in [tRNA^{Ile}]. On the other hand, the $V_{\max}$ so estimated is decreased about fourfold as the KCl concentration is increased to 0.2 M. It should be noted from Figure 4 that the formation of Enz·(Ile-tRNA) is inhibited by salt just as much as the formation of free Ile-tRNA; in no case is there a change in rate at a point corresponding to the synthesis of one molecule of AA-tRNA/molecule of enzyme.

Regardless of how dissociation is measured, it appears to be more rapid at higher ionic strengths. Pingoud et al. (1973) have used stop-flow techniques to show that k_d determined for Enz·(Ser-tRNA) at pH 7.2 increases 12-fold as [KCl] is increased from 0 to 0.2 M. Yarus (1972) has observed a fivefold decrease in K_{ass} for Enz·(Ile-tRNA) at pH 5.5 as KCl is raised to 4 M; much of the decrease must be attributed to an increased k_d. Figure 3 shows that k_d, as measured by the binding assay at 0 °C, increases tenfold as the salt concentration is raised to 0.4 M. We have obtained similar data for the isoleucine enzyme at other temperatures and for the valine system at several temperatures. In every case under every condition, regardless of the method of measurement, the dissociation of Enz·(AA-tRNA) is accelerated by increasing ionic strength. Concurrently, increased ionic strength decreases $V_{\max}$ or has no great effect. These observations cannot be reconciled with the predictions of eq 6; dissociation cannot be rate limiting.

Referring again to eq 6, it is clear that there can be no appreciable increase in $V_{\max}$ unless k_d is increased if $k_d < k_3$. Spermine increases $V_{\max}$ under binding conditions but has no effect on k_d as determined in the binding assay. This too is irreconcilable with the proposition that dissociation of Enz·(Ile-tRNA) is the rate-limiting step.

Our observations with the valine system are very different from those of Hélène et al. (1971). From the binding assay at 20 °C they estimate the k_d for Enz·(Val-tRNA) to be about 0.03 s^{-1} and state without experimental detail that "the rate of release of valyl-tRNA in the presence of a saturating concentration of valine equals the maximal rate of valyl-tRNA formation at the same pH". When we measured the rates of aminoacylation and binding assay "dissociation" under identical conditions (pH 5.5, 20 °C), aminoacylation appeared to be more rapid than "dissociation". When the experiments were conducted at pH 5.0 and 0 °C in order to further slow both reactions, it was clear that the ionic strength effects were similar to those observed for the isoleucine system; salt slowed aminoacylation and accelerated dissociation (Table II). The experiment in Figure 5 shows that, under binding conditions at pH 5.0 and 0 °C, there is no rapid accumulation of Enz·([^{14}C]Val-tRNA). As rapidly as the enzyme·product complex is formed by forward synthesis, it is effectively destroyed by dissociation. Only after most of the substrate tRNA has been converted to Val-tRNA would the amount of bound Val-tRNA be stoichiometrically equivalent to enzyme.

Thus, our reinvestigation of the "binding assay" is not consistent with the proposal that enzyme·product dissociation is rate limiting in the synthesis of Ile-tRNA or Val-tRNA under these conditions. Apart from studies using the "binding assay", the most prominent work supporting the idea of rate-

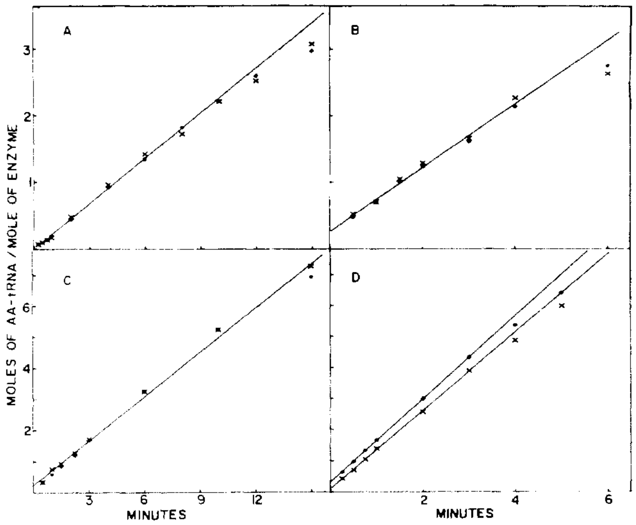

FIGURE 6: Synthesis of aminoacyl-tRNA. Reaction conditions as described in the experimental section except as noted. (A) 20 nM arginine enzyme, 0.5 μM tRNA^{Arg}, 2 mM ATP · Mg, 2 mM excess Mg^{2+}, 10 μM [^{14}C]arginine, 0.1 M sodium cacodylate buffer, pH 6.5, 0 °C. Similar results were obtained at 0 °C and pH 5.5 or 7.5 (Tris). (B) 27 nM isoleucine enzyme, 2.1 μM tRNA^{Ile}, 1.1 mM ATP · Mg, 1.2 mM excess Mg^{2+}, 33 μM [^{14}C]isoleucine, 0.1 M sodium cacodylate buffer, pH 6.0, 0 °C. Similar results were obtained at pH 7.5, 0 °C (Tris) and pH 6.0, 10 °C. (C) 14 μM leucine enzyme, 3 μM tRNA^{Leu}, 1.1 mM ATP · Mg, 1.2 mM excess Mg^{2+}, 75 μM [^{14}C]leucine, 0.1 M sodium cacodylate, pH 7.5, 0 °C. Similar results were obtained at other pH's and at 10 °C. (D) 40 nM valine enzyme, 1 μM tRNA^{Val}, 2 mM ATP · Mg, 0.1 mM excess Mg^{2+}, 10 μM [^{14}C]valine, and 0.1 M sodium cacodylate buffer, pH 5.5, 0 °C. Similar results were obtained in cacodylate, phosphate, Tris, and acetate buffers at all pH's from 5.0 to 8.0 and at temperatures of 0 and 10 °C. All amino acids had specific radioactivities of 35 cpm/pmol. All components of the reaction except one were mixed at reaction temperature for 5 min and reaction was initiated by addition of ATP (X) or tRNA (♦). The data have not been corrected for enzyme blanks, zero-time blanks, etc.

limiting dissociation has been reported by Eldred and Schimmel (1972). They report that the synthesis of Ile-tRNA at pH 6.0 and 0 °C proceeds in two steps. In the presence of excess tRNA^{Ile}, isoleucine enzyme, and either [^{3}H]isoleucyl adenylate or [^{3}H]isoleucine plus ATP, 1 mol of acid insoluble [^{3}H]isoleucine (presumably Ile-tRNA) per mol of enzyme is formed in the first 30–120 s. The second mole of synthesis requires 10–20 min (their Figures 7 and 8). It is difficult to find any interpretation for these biphasic curves except that offered by Eldred and Schimmel, namely that Enz·(Ile-tRNA) is formed rapidly and that the rate of subsequent synthesis is limited by the rate of regeneration of free enzyme. We attempted to find whether there was something unique in the Eldred and Schimmel experiments. Surprisingly, in more than one hundred different experiments conducted under a wide variety of conditions and with four different amino acid:tRNA ligases, we were unable to duplicate the biphasic aminoacylation curve. Figure 6 presents eight such efforts. Regardless of whether the reaction was initiated by final addition of ATP, enzyme, or tRNA, all curves extrapolate without an abrupt change in rate to a point close to the origin.

The formation of Arg-tRNA under conditions like these has previously been reported to be slower than the rate of dissociation of Enz·(Arg-tRNA) (Evans and Nazario, 1974), so it was expected that the synthesis of Arg-tRNA would be linear with time. However, the syntheses of Val-tRNA and Ile-tRNA have been stated to be limited by the rate of product dissociation (Eldred and Schimmel, 1972; Yarus and Berg 1969; Hélène et al., 1971), so these should show the biphasic behavior found by Eldred and Schimmel. It has been implied that the synthesis of Leu-tRNA is also limited by product dissociation (Rouget and Chapeville, 1971); a biphasic curve might have been expected here too.

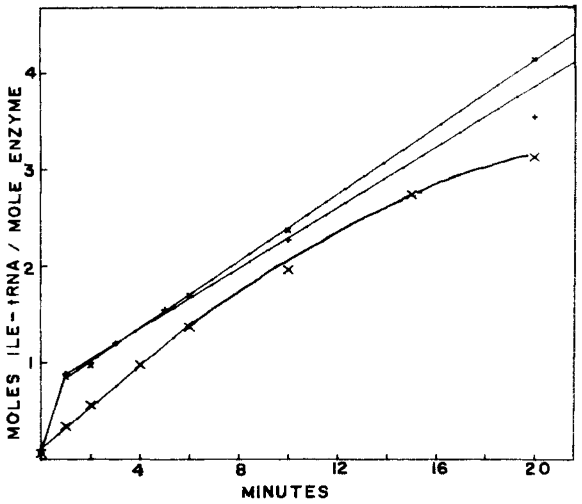

FIGURE 7: Formation of Ile-tRNA pH 5.5, 0 °C. Conditions and methods as described except that the experiment was conducted in a room with 22 °C ambient temperature. For (+), 0.48 μM tRNA^{Ile} and (x), 2.4 μM tRNA^{Ile} aliquots were removed with a 45-μl glass micropipet at room temperature. (X) 0.48 μM tRNA^{Ile}, conditions identical except that the pipets were precooled to 0 °C.

Enzyme, amino acid, and ATP were present together for 5 min in those reactions that were initiated with tRNA. Presumably Enz·(AA~AMP) was present when tRNA was added, but this did not result in a rapid burst of synthesis of AA-tRNA in any case. Enzyme, amino acid, and tRNA were together for 5 min in reactions initiated with ATP, but the prior association of Enz·tRNA·AA did not affect the kinetics.

With all four enzyme systems, we find linear aminoacylation of tRNA at 0 °C and at all pH's from 5.0 to 7.5 and at all ionic strengths. Naturally when the supply of tRNA is small, the reaction curve flattens as a result of substrate exhaustion. With higher concentrations of tRNA, the reaction is more nearly linear for a longer time but some bowing is noticeable, possibly due to a combination of product inhibition, deacylation, and substrate depletion (e.g., Figures 1B and 4).

At temperatures higher than zero, this type of kinetic study becomes difficult because of the much higher reaction rates. Nonetheless, we find that the reactions are linear under all conditions at 10 °C with at least one observation possible before 1 mol of AA·tRNA has been formed per mol of enzyme. At still higher temperatures no observations are possible during the first cycle of synthesis, but the earliest data points extrapolate towards the origin (e.g., Figure 1B).

All the above experiments were conducted in a cold room using baths of appropriate temperature. When the formation of Ile-tRNA was reexamined at 0 °C but not in the cold room, we observed biphasic kinetics (Figure 7). We suspected that the rapid initial rise was due to the fact that 45-μl aliquots had been withdrawn into pipettes at 22 °C. The small amount of fluid at 0 °C was warmed briefly by the glass pipet before it was expressed into trichloroacetic acid. Perhaps during the few seconds at the higher temperature there was as much reaction as would have occurred in a minute at 0 °C. The experiment was repeated exactly, in an ice bath in the open laboratory. However, the aliquots were withdrawn into pipets precooled to 0 °C. Under these conditions the reaction is monophasic, as shown by the lower curve of Figure 7. We have determined by the use of a microthermistor that water at 0 °C when drawn into a 45-μl glass micropipet at 22 °C has attained a temperature of 16.5 °C by the time it is blown out 6 s later. (Water at 0 °C was about 1 °C after being drawn into and blown out of a pipette precooled to 0 °C.) Since the aminoacylation reactions are about 15 times faster at 17 than at 0 °C, "our artifact" accounts for a burst of 0.75 mol of product/mol of enzyme. Eldred and Schimmel (1972) pipetted aliquots of the reaction mixture onto filter paper pads that had been pretreated with trichloroacetic acid. If interaction of the enzyme with trichloroacetic acid is not instantaneous, this would contribute to the appearance of an initial burst.

Carr et al. (1975) describe an experiment in which the rate of formation of Ile-tRNA at 37 °C, pH 8.0, is measured. They extrapolate back to zero time from observations at 5 min and at 45 s. They conclude that there has been a burst of synthesis like that observed by Eldred and Schimmel. Since the aminoacylation curves usually become concave as substrate is consumed and product is formed, we feel the extrapolation based on two points is tenuous.

The present work establishes that at low pH and low temperature the rate of aminoacylation is not limited by the rate of dissociation of product from enzyme. Since these reaction conditions are far from physiological, this conclusion might ordinarily be considered trivial. However, the only evidence suggesting that enzyme·product dissociation is rate-limiting has been derived from experiments conducted at low pH and low temperature. Yarus and Berg (1969) studied the activation energy for the formation of the Ile-tRNA at pH 5.5 and 7.5 from 0 to 38 °C, and from this argued that there is no change with temperature or pH in the mechanism of the reaction or in the nature of the rate-limiting step. If we accept their arguments, we must conclude that dissociation of enzyme·product complex is not rate limiting at higher pH and higher temperature. Experiments at physiological pH and 10 °C with four enzyme systems are inconsistent with a slow rate-determining dissociation of product (similar to Figure 6). All recent work with other enzyme systems in other laboratories is in agreement with this conclusion. Krauss et al. (1973) state for the phenylalanine enzyme of yeast (pH 7.2): "the rate-limiting step in the entire process of aminoacylation occurs before the release of the acylated tRNA from the enzyme". Pingoud et al. (1973) found that, at pH 7.2, the rate of dissociation of Enz·(Ser-tRNA) is about 60 times faster than the rate-limiting step in the synthesis. Evans and Nazario (1974) find that at pH 5.5 (binding conditions), formation of Enz·(Arg-tRNA) is 30 times slower than dissociation. Fasiolo and Ebel (1974) observe that (pH 6.0, 0 °C) Enz·(Phe-tRNA) is dissociated more quickly than it forms. Fersht and Jakes (1975) and Fersht and Kaethner (1976) find no "burst" in the synthesis of either Tyr-tRNA or Ile-tRNA at 25 °C and pH 7.7 or 5.87. They conclude that enzyme·product dissociation cannot be rate limiting in either condition.

Our experience and a consideration of published data lead us to question whether the binding assay accurately measures only the dissociation of Enz·(AA-tRNA). Using this method, Rouget and Chapeville determined k_d for Enz·(Leu-tRNA) to be 0.008–0.05 s^{-1} (pH 6.0, 20 °C) (there may be errors in their calculation). We find k_3 under similar conditions to be about 0.2 s^{-1}. From the binding assay method, Hélène et al. (1971) obtained a value of 0.004–0.04 s^{-1} for the dissociation of Enz·(Val-tRNA) (pH 5.5, 20 °C), while we find k_3 to be about 0.45 s^{-1}. In the absence of added salt, the measured "dissociation" of Enz·(Ile-tRNA) can be six times slower than k_3, as noted in Table I.

Since it is obvious that the overall rate of aminoacylation of tRNA cannot be greater than the rate of dissociation of product from enzyme, one must conclude from these examples that "k_d's" measured by the binding assay are not merely the rates of dissociation of the Enz·(AA-tRNA) complex. Yarus and Berg (1969) have pointed out that, since the semilogarithmic plot yields a straight line, "the system is homogeneous and has only one rate of exchange". In most of our experiments and in some published experiments (i.e., Yarus and Berg (1969), Figures 2 and 5; Yarus (1972), Figure 9; Rouget and Chapeville (1971), Figure 13) the data are not ideal. In many cases the best fitting exponential or logarithmic curve intersects with the ordinate below the observed zero time data. This suggests that there may be some small fraction of the complex that exchanges rapidly. Furthermore, in many cases the reaction does not approach the theoretical equilibrium. These deviations from ideality suggest that the system is not homogeneous. There is, without doubt, a highly specific association of $tRNA^{Ile}$ with the isoleucine enzyme, but quite possibly this association and its reversal are rapid compared to the rates of subsequent reversible conformational changes or to the rates of reversible enzyme aggregation. Yaniv and Gros (1969) have demonstrated Enz·(Val-tRNA) complexes by ultracentrifugation at pH 5.5. They attribute their low recovery of enzyme activity to a tendency of the enzyme to form high-molecular-weight aggregates. Yarus and Berg (1967, 1970) observe that the enzyme·tRNA complex, as retained on the filter, lacks enzymatic activity and no longer releases its tRNA easily. It is possible that only those enzyme·tRNA complexes that have undergone slight changes in conformation or aggregation (and loss of enzymatic activity) can be bound to the filter. Those enzyme·tRNA complexes whose dissociation has been measured directly (at pH 7.2) have k_d's hundreds or thousands of times greater than the estimates obtained with this assay at pH 5.5 (Krauss et al. (1973); Pingoud et al. (1973)).

Using a technique quite different from the binding assay, Eldred and Schimmel (1972) report that the dissociation of Enz·(Ile-tRNA) in the absence of effectors is 2×10^{-4} s^{-1} at pH 6.0, 3 °C. This extremely slow rate was confirmed qualitatively by still another method (Eldred and Schimmel, 1973) and is substantially slower than the value reported here (1.1×10^{-3} at pH 5.5 and 0 °C). In both of Eldred and Schimmel's experiments, the Enz·(Ile-tRNA) was generated by reaction of Enz·(Ile~AMP) with tRNA. Is it possible that different routes to preparation result in slightly different conformations that dissociate at different rates? Along the same line, it should be noted that most of the experiments that suggest dissociation is slow have been conducted with either preformed AA-tRNA or AA-tRNA synthesized on the enzyme from AA~AMP. At least one material ordinarily present in the aminoacylation reaction is missing, pyrophosphate.

The present work and other work cited above exclude enzyme·product dissociation as a rate-limiting step in the aminoacylation of tRNA. From the present study, it is not possible to identify the rate-limiting step. As with most enzymatic reactions, the overall rate appears to depend on both the availability of enzyme·substrate complex and the rate (k_3) at which this reacts to yield product. Since increased ionic strength has either no effect or only a mildly inhibitory effect on k_3, the principal consequence of adding salt is to decrease the rate or the extent of formation of a complex containing enzyme, tRNA, and other substrates (Loftfield and Eigner, 1967).

Appendix: Derivation of Equations 1 and 2

$$\text{Enz} + \text{AA} + \text{ATP} \xrightarrow[\text{fast}]{\text{very}} \underset{\text{I}}{\text{Enz} \cdot (\text{AA}{\sim}\text{AMP})} + \text{PP}_i$$

$$\mathrm{I} + \underset{\mathrm{S}}{\mathrm{tRNA}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{\mathrm{II}}{\mathrm{Enz}\cdot(\mathrm{AA{\sim}AMP})\cdot\mathrm{tRNA}}$$

$$\xrightarrow{k_3} \underset{\mathrm{III}}{\mathrm{Enz}\cdot(\mathrm{AA\text{-}tRNA})} + \mathrm{AMP}$$

$$\xrightarrow{k_{\mathrm{d}}} \underset{\mathrm{P}}{\mathrm{AA\text{-}tRNA}} + \mathrm{Enz}\ \text{(which reacts very fast in step I)}$$

$$v = \frac{\mathrm{d}P}{\mathrm{d}t} = k_{\mathrm{d}}(\mathrm{III})$$

$$\frac{\mathrm{d(III)}}{\mathrm{d}t} = k_3(\mathrm{II}) - k_{\mathrm{d}}(\mathrm{III}) = 0$$

$$\frac{\mathrm{d(II)}}{\mathrm{d}t} = k_1(\mathrm{I})(\mathrm{S}) - (k_2 + k_3)(\mathrm{II}) = 0$$

$$(\mathrm{III}) = \frac{k_3(\mathrm{II})}{k_{\mathrm{d}}}$$

$$(\mathrm{II}) = \frac{k_1(\mathrm{S})(\mathrm{I})}{k_2 + k_3} = \frac{(\mathrm{S})(\mathrm{I})}{K_{\mathrm{m}}}$$

statement of conservation: $(\mathrm{E_0}) = (\mathrm{I}) + (\mathrm{II}) + (\mathrm{III})$: free enzyme is negligible or $(\mathrm{I}) = (\mathrm{E_0}) - (\mathrm{II}) - (\mathrm{III})$

$$(\mathrm{I}) = (\mathrm{E_0}) - \frac{(\mathrm{I})(\mathrm{S})}{K_m} - \frac{k_3}{k_{\mathrm{d}}}\frac{(\mathrm{I})(\mathrm{S})}{K_m}$$

$$(\mathrm{I}) + \frac{(\mathrm{I})(\mathrm{S})}{K_m} + \frac{k_3}{k_{\mathrm{d}}}\frac{(\mathrm{I})(\mathrm{S})}{K_m} = (\mathrm{E_0})$$

$$(\mathrm{I})\left(1 + \left[1 + \frac{k_3}{k_{\mathrm{d}}}\right]\frac{(\mathrm{S})}{K_{\mathrm{m}}}\right) = (\mathrm{E_0})$$

$$(\mathrm{I}) = (\mathrm{E_0})\Big/\left[1 + \frac{(\mathrm{S})}{K_{\mathrm{m}}}\left(1 + \frac{k_3}{k_{\mathrm{d}}}\right)\right]$$

$$v = k_{\mathrm{d}}(\mathrm{III}) = k_3(\mathrm{II}) = k_3\frac{(\mathrm{I})(\mathrm{S})}{K_{\mathrm{m}}}$$

$$= \frac{k_3(\mathrm{S})(\mathrm{E_0})}{K_{\mathrm{m}}\left(1 + \frac{(\mathrm{S})}{K_{\mathrm{m}}}\left(1 + \frac{k_3}{k_{\mathrm{d}}}\right)\right)}$$

$$v = \frac{k_3(\mathrm{S})(\mathrm{E_0})}{K_{\mathrm{m}} + (\mathrm{S})(1 + k_3/k_{\mathrm{d}})}$$

$$\text{as } (\mathrm{S}) \gg K_{\mathrm{m}},\ v = V_{\max} = \frac{k_3(\mathrm{S})(\mathrm{E_0})}{(\mathrm{S})(1 + k_3/k_{\mathrm{d}})} = \frac{k_3(\mathrm{E_0})k_{\mathrm{d}}}{k_{\mathrm{d}} + k_3}$$

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